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Abstract

Purpose: Abdominal wall hernia is one of the commonest surgical disorders world-wide and there is no single gold-standard operative technique to repair it. In an effort to improve techniques and technologies to reinforce hernia repair, synthetic meshes are employed. In this study a new prosthesis (named composite) formed of two polypropylene layers, one macroporous (named mesh) and one transparent (named film) was examined to evaluate its capability to enable cell proliferation without inducing cell death. Inflammatory processes were also examined.

Methods: Human fibroblasts BJ were seeded on multiwells, on which composite or film had been placed. After 7, 14 and 21 days, cell growth and viability, deposition of collagen, and release of IL-6, IL-1β and TNF-α were evaluated.

Results: The "in vitro" protocol showed the composite to be colonized by human fibroblasts on the polypropylene macroporous mesh side; no cell growth occurred on the film. The slowdown of cell growth observed between 14 and 21 days was accompanied by an increase of type I collagen deposition and marked fibroblast activity. Inflammatory cytokines initially increased, followed by their reduction beginning at 14 days.

Conclusions: The new prosthesis comprising two polypropylene layers of differing morphologies can be colonized by fibroblasts on the side facing the abdominal wall, whereas no cell growth occurs on the side facing the viscera. The transient inflammation, observed at early experimental times, is probably important for the healing process.

Introduction

Abdominal wall hernia is one of the commonest surgical conditions world-wide and there is no single gold-standard operative technique for repairing it [1]. Despite significant advances in techniques and technologies, recurrence rates following standard ventral herniorrhaphy remain unacceptably high [2].

In an effort to reduce recurrence rates, synthetic meshes are employed, and their introduction was a milestone in hernia repair [3]. The ideal mesh should have no adhesion potential, excellent tissue integration, minimal shrinkage, and should be easy to use. Moreover, it should not promote infection, fistula, or seroma formation and should not limit or negatively affect the patient's normal activity [4].

Millions of meshes are implanted every year, and they have unquestionably been demonstrated to ensure tension-free mechanical stability and fascial reinforcement in the repair of hernial defects of the abdominal wall, with very low recurrence rates [5]. With respect to oncogenic potential, findings are sufficient to conclude that modern mesh materials and construction are not associated with an increased risk of malignant tissue degeneration [6]. However, some other implant-determined postoperative problems, such as chronic pain, foreign-body sensation, and the phenomenon of 'stiff abdomen' remain unsolved, and it is clear that they are due to the material and construction characteristics of the implants.

At present synthetic meshes are most often categorized as macroporous, microporous, or composite [7]. Macroporous meshes include polypropylene-monofilament and polyester-multifilament meshes, among many others [8]. These macroporous meshes consist of two subgroups: those with large pores (most of them are the so-called heavyweight meshes) and those with very large pores (most of them are the so-called lightweight meshes). Heavyweight

meshes have greater tensile strength than lightweight meshes, but cause a more intense foreign-body reaction, produce greater shrinkage than lightweight meshes, and are stiffer [9]. Lightweight meshes are designed to mimic the physiology of the abdominal wall, and their tensile strength is adapted to that of tissue [10]. When placed in contact with abdominal viscera, however, macroporous meshes are associated with the formation of bowel adhesions and obstructions, and enterocutaneous fistulae [11]. Therefore, these meshes should be avoided or used in combination with vascularized tissue (e.g., greater omentum, hernia sac) or antiadhesive barriers when contact with the bowel is likely. On the other hand there are microporous meshes or films, such as ePTFE (expanded polytetrafluoroethylene), that have a smaller pore size that does not allow tissue in-growth, but may lead to encapsulation and persistence of bacteria. Thus microporous meshes have a lower risk of adhesions, but may be more susceptible to infection.

For laparoscopic ventral hernia repair, the mesh composition must form an adequate barrier between the viscera and the abdominal wall. A wide variety of composites are now available that combine different qualities, having for example macroporous mesh on one side to promote tissue in-growth and microporous mesh on the other side to reduce the risk of adhesions (e.g., polypropylene/ePTFE). Synthetic meshes with antiadhesive coatings have also been developed. Such coatings include non-absorbable (e.g., titanium, polyurethane) and absorbable coatings (e.g., omega-3 fatty acid, collagen hydrogel, oxygenated regenerated cellulose, polydioxanone, polyethylene glycol). Clinical evidence suggests that composite and coated synthetic meshes carry a reduced risk of adhesions compared with traditional synthetic meshes [12,13]. The relative benefits of these different prostheses with regard to adhesion formation and risk of infection vary with different study models and methodologies, and outcomes also differ [14]. To avoid any undesired local or systemic side-effects, an initial

preclinical evaluation of newly developed mesh constructions is required, to evaluate their biological response.

In this study an "in vitro" model was used to evaluate the capability of a new prosthesis to allow cell proliferation, without inducing cells to die by apoptosis or necrosis, in the parts of the prosthesis in contact with the abdominal wall, and to prevent cell proliferation in parts in contact with the viscera, and not produce inflammatory factors. The new prosthesis is composed of two polypropylene layers, one macroporous mesh and one transparent film. Human fibroblasts were used to test it because this type of cell is in constant contact with the foreign material during the process of scar formation, and plays a leading role, acting as an important growth-factor-producing defensive cell barrier [15].

Methods

Prosthesis preparation

The new prosthesis is comprised of two polypropylene layers, one macroporous light mesh, and one thin transparent film (CMC, Clear Composite Mesh, DIPROMED srl S.Mauro Torinese –Turin, Italy).The two layers possess different properties in order to best perform their functions. The mesh for the parietal side is macroporous with a 88% of porosity and 45gr/m^2; it is made of polypropylene monofilament 120 μm of diameter, in order to optimize tissue growth. The film for visceral side is composed by non-porous, smooth and transparent polypropylene with a thickness of 50μm (Type IV) [8] in order to prevent the adhesion formation in the intestinal side [16] (Figure 1).

The company designed the device by selecting a type of weave that would to meet the requirements of lightness, softness, high stability and porosity.

The CMC is much thinner than other prostheses on the market; the transparency of the polypropylene film enables viewing of blood vessels, nerves, and underlying tissues during placement of the prosthesis. The polypropylene mesh and the polypropylene film are sewn together.

Human fibroblast culture conditions

Human fibroblasts BJ (ATCC, Rockville, MD, USA) were cultured (33,000/cm^2) in DMEM high glucose medium supplemented with 2 mM glutamine, 1% antibiotic/antimycotic solution, 1% not-essential amino acids, and 10% FBS.

Fibroblasts were seeded on multiwells, where the CMC (2.5 cm x 2.5 cm) or film of equal size had been placed and anchored using biologically-inert sterile stickers, and on multiwells without composite or film as controls. After 7, 14 and 21 days, the following analyses were carried out.

Light microscope analysis

After removing culture medium from each well, CMC or film colonized by BJ cells were observed under light-reverted microscope.

Cell growth and viability

Cells were detached from CMC or film by trypsinization. Cell growth was determined by counting the trypsinized cells in a Burker chamber under a light microscope (Leitz, Wetzlar, HM-LUX, Germany).

Cell viability was evaluated by determining lactate dehydrogenase release in the culture medium and DNA content on fixed cells by cytofluorimetric assay [17].

Immunohistochemistry analysis

To evaluate the production of type I collagen by fibroblasts colonizing the CMC, ABC Staining System and monoclonal anti-collagen 1A1 antibody (Santa Cruz Biotechnology, INC, Germany) were used.

Cytokine analysis

IL-6, IL-1β and TNF-α were evaluated using Enzyme Linked Immuno Sorbent Assay (Bender Medsystems, Vienna, Austria) in the culture medium of fibroblasts colonizing the CMC or growing without CMC, considered as controls. The content of cytokines was normalized to the $1x10^6$ of cells.

Statistical analysis

All data are expressed as means ± S.D. Differences between group means were assessed by analysis of variance followed by a *post-hoc* Newman-Keuls test.

Results

Figure 2 shows the microscopic appearance of CMC before fibroblast BJ seeding. As shown in Figures 3, at different experimental times fibroblasts were able to grow on the composite and to colonize both mesh and film components. It is clear from the Figure that the number of fibroblasts present on mesh filaments and film increased throughout the experiment. On the contrary, cells seeded on the film alone without the macroporus mesh did not grow during the experiment (images not shown).

The behaviour over time of BJ cells on the composite, and on the film alone, was confirmed by counting cells after detaching them by trypsin (Figure 4). On the CMC, cells had increased 1.5, 2.4 and 2.6 times at 7, 14 and 21 days, respectively. These increases were similar to those

of cells grown without CMC or film (control cells) at 7 and 14 days, whereas at 21 days the number of control cells was higher than the cells on CMC. Figure 4 also shows numbers of fibroblasts growing on the isolated film: the number was in all cases lower than that of cells present on the CMC and of control cells; it did not increase over time, evidencing the inability of cells to grow in the absence of mesh. Cell viability was evaluated as the release of lactate dehydrogenases in the culture medium and as DNA content determined through cytofluorimetric analysis. The determination of lactate dehydrogenase release showed that no cells died by necrosis, in the presence or absence of either composite or film alone (data not shown). Figure 5 reports cell viability evaluated by cytofluorimetry. The majority of the control and CMC cells were viable at all experimental times, whereas the majority of cells detached from the film had died by apoptosis, with the exception of the 7-day time-point.

To determine fibroblast activity, type I collagen was evaluated by immunohistochemistry analysis (Figure 6 and 7). It is evident that the deposition of collagen on the composite increased during the experimental time-frame.

To rule out the hypothesis that the prosthesis used in this research could induce inflammatory processes, the release of cytokines in the medium from the cells cultured on CMC and compared with that from cells cultured without CMC, was determined. The release of cytokines in the medium from the cells cultured on film was not evaluated since no cell growth was evidenced during the experimental times. Figure 8 shows that the content of IL-6, IL-1β and TNF-α was higher in CMC than in control at 7 days of treatment. In the following experimental times (14 and 21 days) the content of IL-1β and TNF-α decreased in CMC compared to 7 days. On the contrary, the content of IL-6 remained unchanged at 14 days, further increasing at 21 days in the presence of the CMC compared to control cells.

Discussion

The development of mesh for successful hernia repair is an intricate and difficult task. The use of mesh implants in hernia surgery has become increasingly widespread, due to good postoperative results and low rates of long-term recurrence [5,18]. Polypropylene has been established as the basic synthetic material for mesh; it is a versatile thermoplastic polymer that exhibits excellent chemical resistance, low density, high tensile strength and a relatively high melting point, especially in comparison to its counterpart, polyethylene.

Before using any new prosthesis, even if investigated and designed with new technologies, it is essential to examine it in animal and "in vitro" models, in order to evaluate its biocompatibility.

In this study, an "in vitro" model was used to determine whether the new prosthesis may be regarded as an ideal mesh. Polypropylene is used for both sides of the CMC (lightweight mesh on the parietal side and thin transparent film on the visceral side) because the macroporous mesh promotes tissue in-growth, and the smooth film withstands adhesion formation due to its plane surface. The woven polypropylene is designed for tissue in-growth for adequate prosthesis integration, due to its 2D scaffold feature which improves cell proliferation. On the other side, the polypropylene film provides a surface that is adequate to prevent adhesion formation. In fact, the cells seeding on the film did not proliferate and as consequence they underwent apoptosis, a physiological death. The surface morphology influences interactions and foreign-body reactions, and is thus determinant for successful implant outcome.

The "in vitro" analysis of cell cultures (as a model of single-cell organisms) offers the opportunity to systematically study the biological response, avoiding the interference from other extracellular influences on growth; separate immunological reactions can be identified and compared with control cells experiencing normal growth processes. For this reason, one-

dimensional “in vitro” models should be required as first-line experimental design for biomaterial research [19].

The “in vitro” analyses used in this study showed that the new prosthesis, comprising the two layers, one of polypropylene macroporous lightweight mesh, and the other a thin transparent polypropylene film, can be colonized by human fibroblasts BJ on the side facing the abdominal wall, whereas the cells did not grow on the other side.

Fibroblasts were chosen for the study because of their basic role in the processes of wound healing and because they are in constant contact with the foreign material in the process of scar formation and play a leading role as an important growth factor-producing defensive cell barrier. [20,21]

The good growth of fibroblasts on the film on the side with macroporous mesh was probably due to the presence of the mesh, since there was no grow of the cells on the film alone. This observation may be indirectly confirmed by the results of our previous study, showing that the surface properties of oral implants induce osteointegration differently: ossification begins later at the surface of machined implants than porous ones. Thus the presence of a non-smooth surface allowed better colonization by cells [22].

The slowdown of cell growth on the CMC observed between 14 and 21 days was accompanied by an increase of type I collagen deposition, indicating that the CMC also enables the principal function of fibroblasts to continue. At the moment, the type I collagen deposition was evaluated only qualitatively, since the mesh presence made difficult quantitative evaluation.

The increased production of cytokines IL-6, IL-1β and TNF-α in the culture medium in the presence of prosthesis in comparison with cells grown in absence, at the first experimental time-point (7 days), was indication that the presence of prosthesis can induce an inflammatory process. At the moment, it is not clear if this increase was due to the morphology of CMC or

to the type of material (polypropylene). The cytokine production was decreasing at the following time-points (14 and 21 days), as evidenced by IL-1β and TNF-α decrease and by the increase of collagen. The early and transient induction of inflammatory processes is probably important in favouring healing process.

The fact that IL-6 remained high in all experimental times is not unusual, because this cytokine is known for having both pro- and anti-inflammatory properties. In fact, IL-6 could facilitate wound healing, for its slight anti-inflammatory activity [23,24]: it down-regulates the synthesis of IL-1 and TNF and have little effect on the synthesis of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) [24].

In conclusion, it may be said that the new prosthesis, comprising two polypropylene layers with different morphology, one layer formed of macroporous mesh and the other of transparent film, can be easily colonized by fibroblasts, inducing their activity of producing collagen.

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Conflict of interest

All the authors declare that no conflict exists

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Figure legends

Figure 1

Prosthesis project

Figure 2

Light-reverted microscope analysis of the polypropylene CMC (mesh + film) before cell seeding.

Figure 3

Light-microscope analysis of fibroblasts present on the CMC (mesh + film) at 7, 14 and 21 days after cell seeding.

Figure 4

Number of fibroblasts present on CMC or on film alone at different experimental times.

Data are means ± S.D. of 4 experiments. Means with different letters are significantly different from one another ($p<0.05$) as determined by analysis of variance followed by *post-hoc* Newman-Keuls test.

C, control cells (without CMC or film)

CMC, mesh + film

Figure 5

Viability of fibroblasts present on composite or film alone at different experimental times.

Data are means ± S.D. of 4 experiments and are expressed as percentages of viable or dead cells versus total cells. For each panel, means with different letters are significantly different

from one another ($p<0.05$) as determined by analysis of variance followed by *post-hoc* Newman-Keuls test.

C, control cells (without CMC or film)

CMC, mesh + film

Figure 6

Immunohistochemistry analysis of type I collagen produced by fibroblasts present on the composite 7 and 14 days after cell seeding.

Figure 7

Immunohistochemistry analysis of type I collagen produced by fibroblasts present on the composite 21 days after cell seeding.

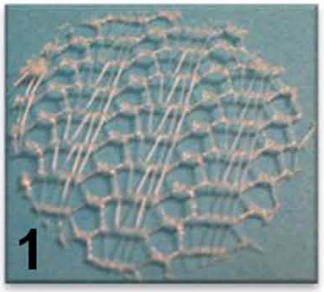
Figure 8

Content of cytokines IL-6, IL-1β and TNF-α in the culture medium of fibroblasts, grown in the presence or absence of CMC.

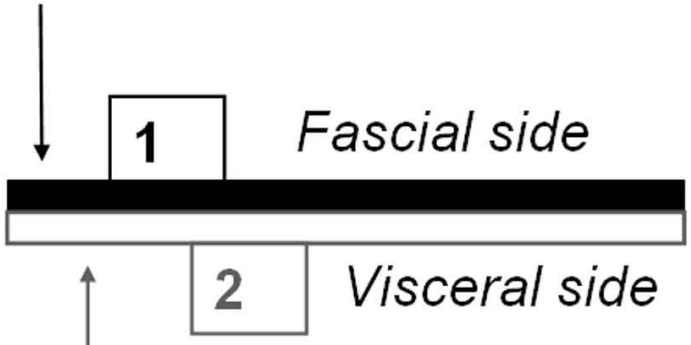
Cytokine content was evaluated in the medium of fibroblasts grown in the presence (CMC) or in absence of CMC (C) and the values were normalized to $1x10^6$ cells. Data are means ± S.D. of 4 experiments, and are expressed as percentage of control (C), taken as 100. For each panel, means with different letters are significantly different from one another ($p<0.05$) as determined by analysis of variance followed by *post-hoc* Newman-Keuls analysis.

Figure 1

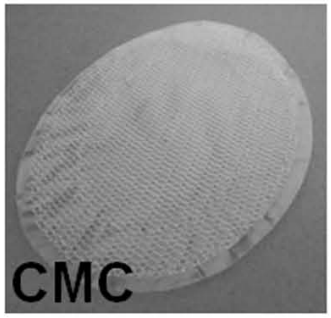
Prosthesis project



Macroporous mesh for cell ingrowth

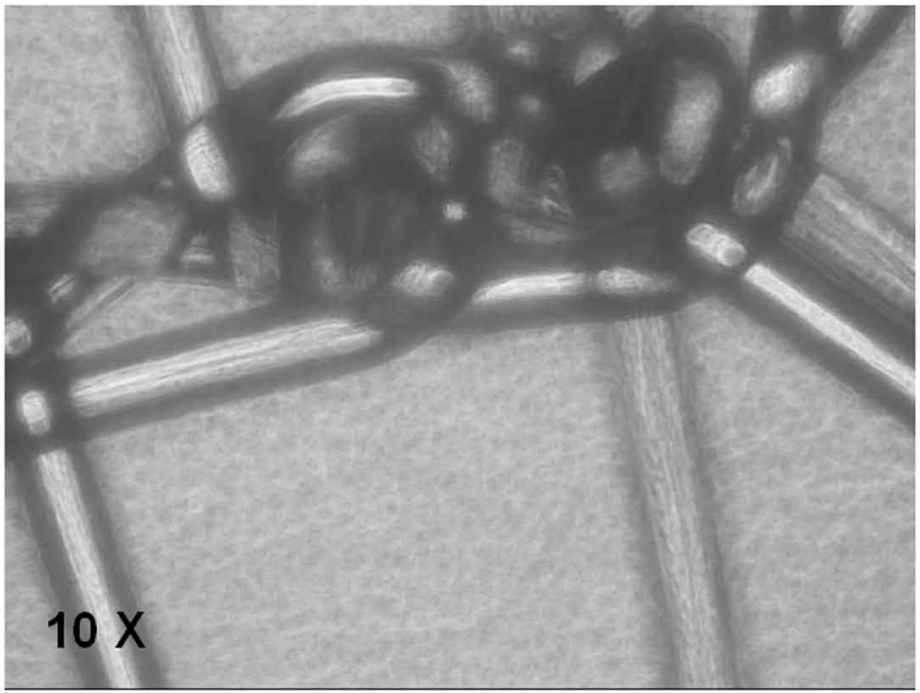


Non-porous smooth trasparent film with antiadhesive properties



Clear Composite Mesh = CMC

Figure 2



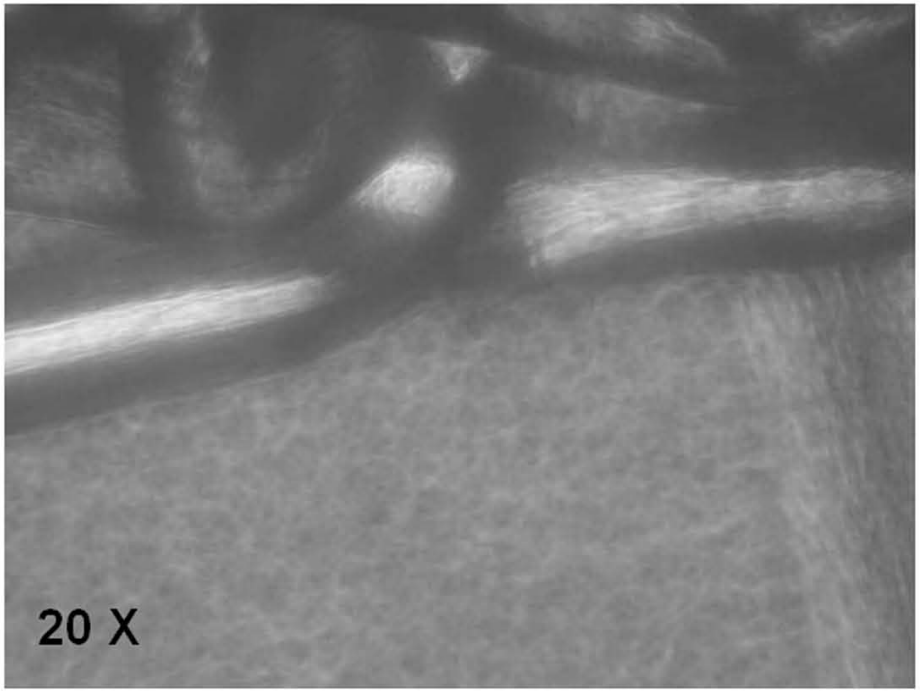
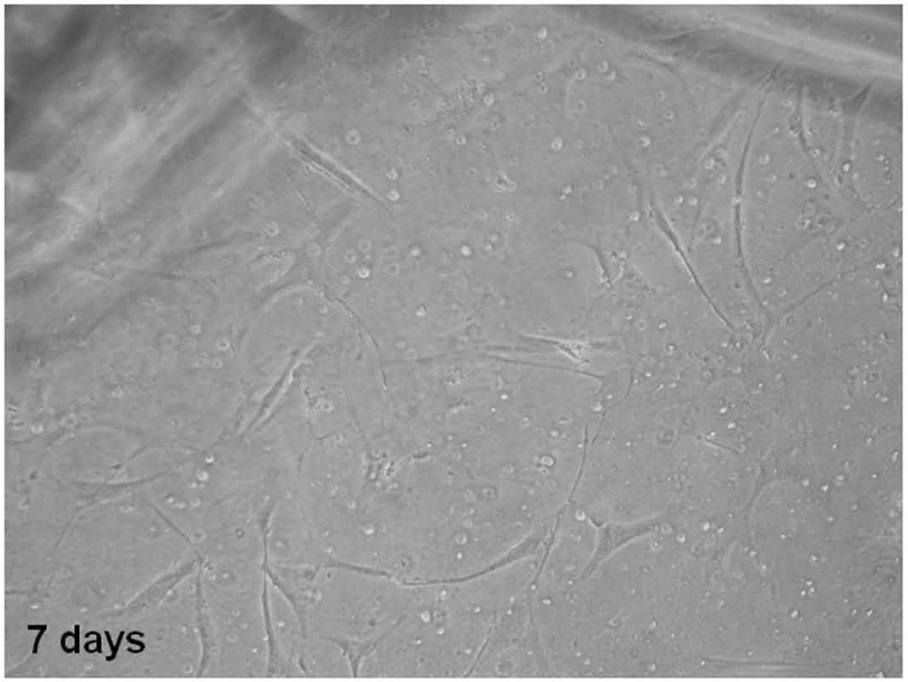
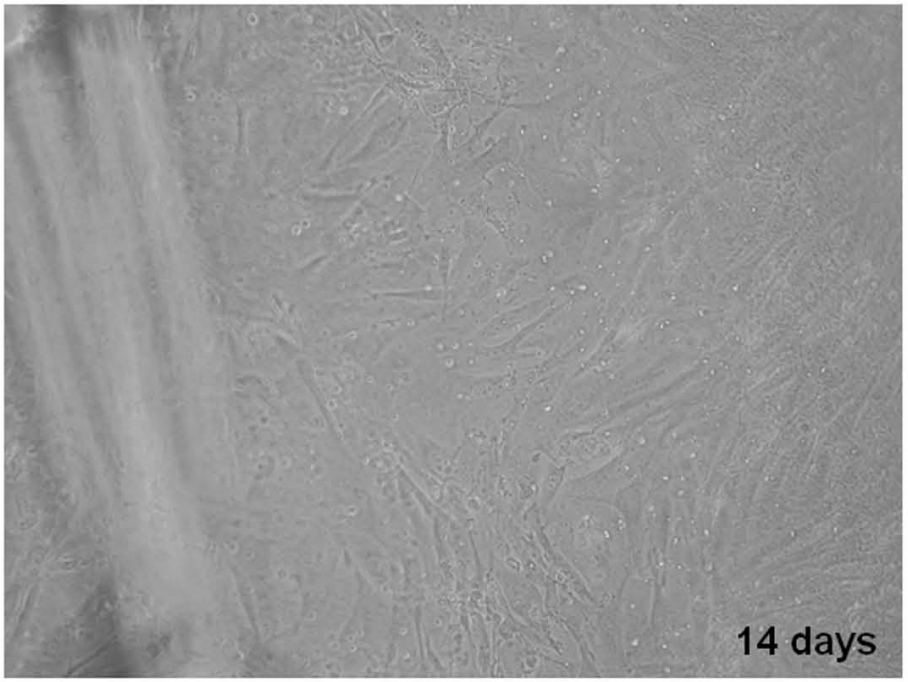


Figure 3





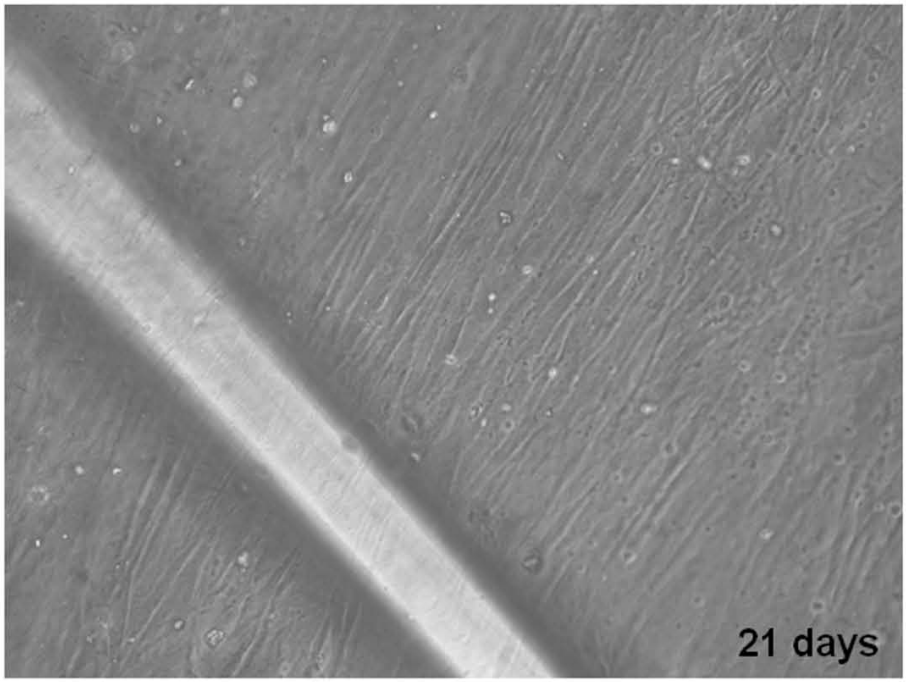
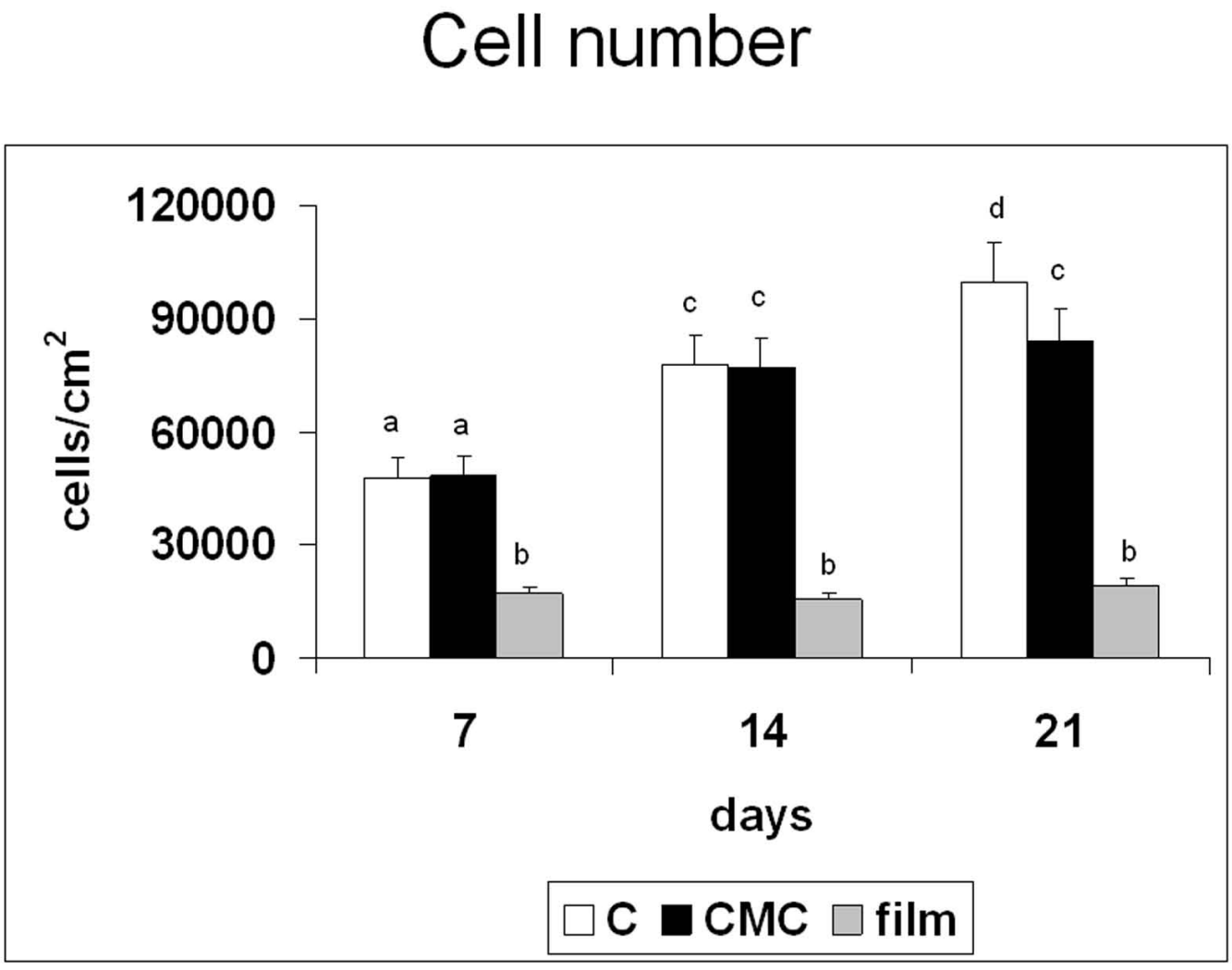


Figure 4


Cell number
120000
90000
60000
30000
0
cells/cm2
a
a
b
c
c
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b
7
14
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days
C
CMC
film

Figure 5

Cell viability

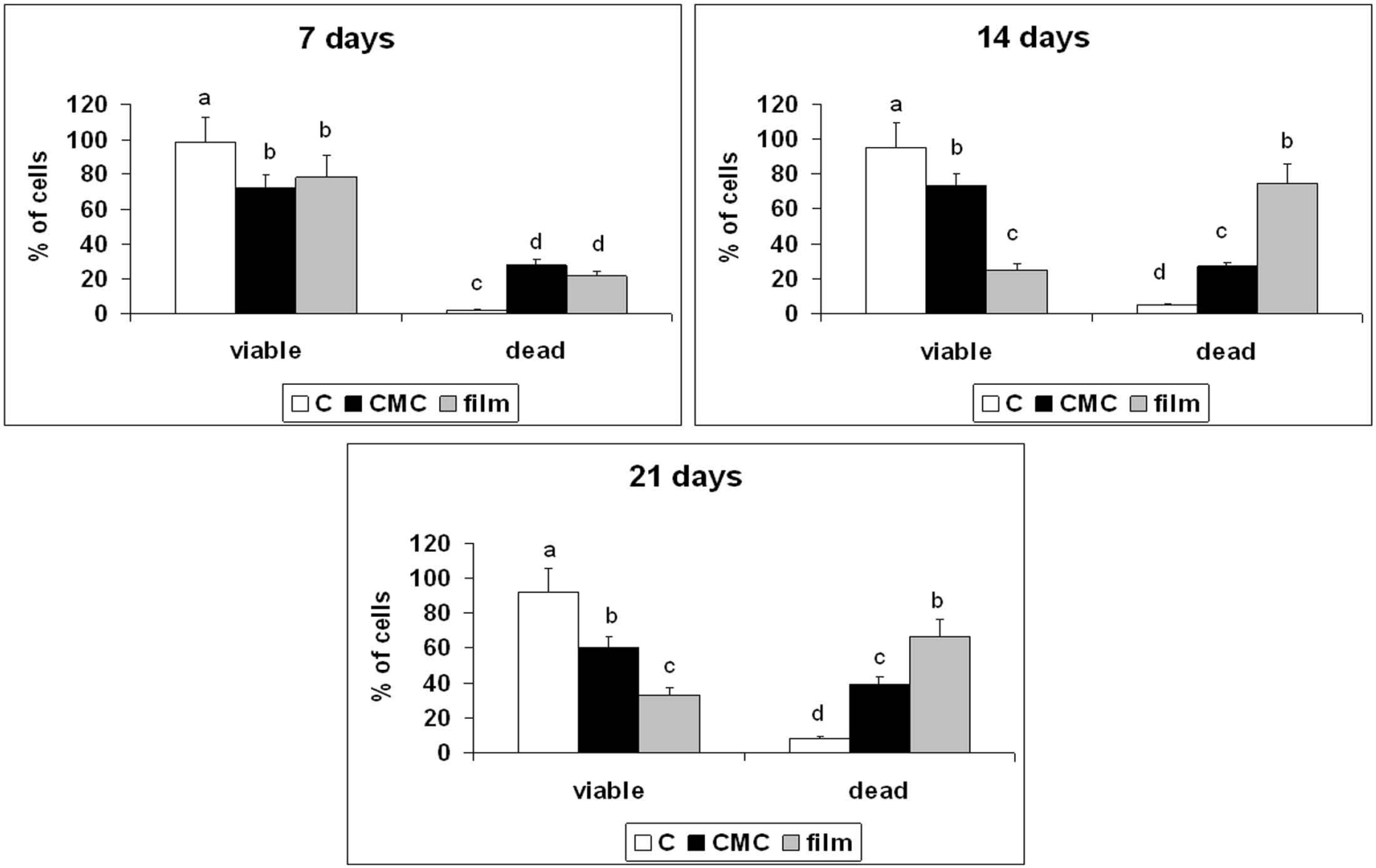


Figure 6

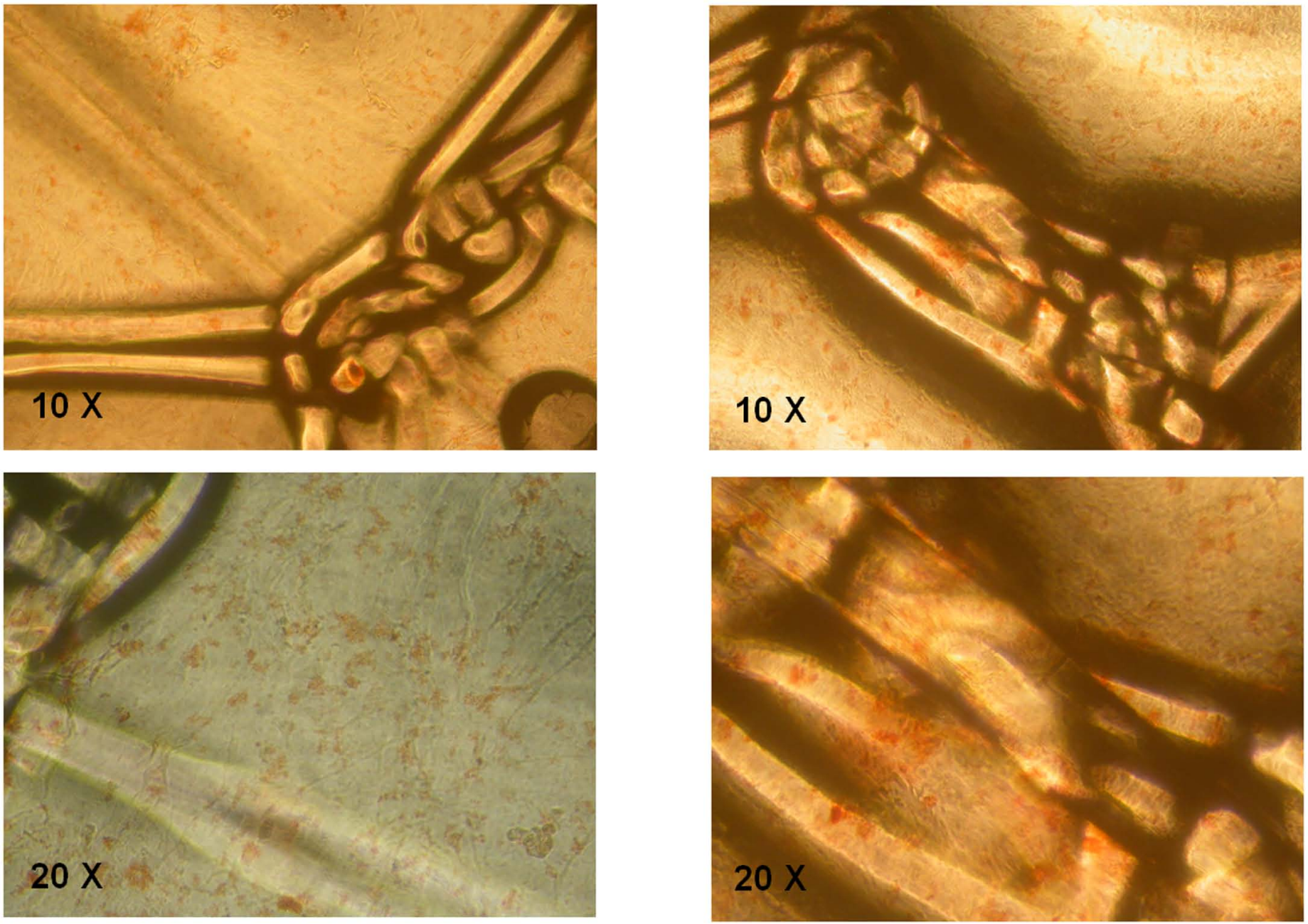
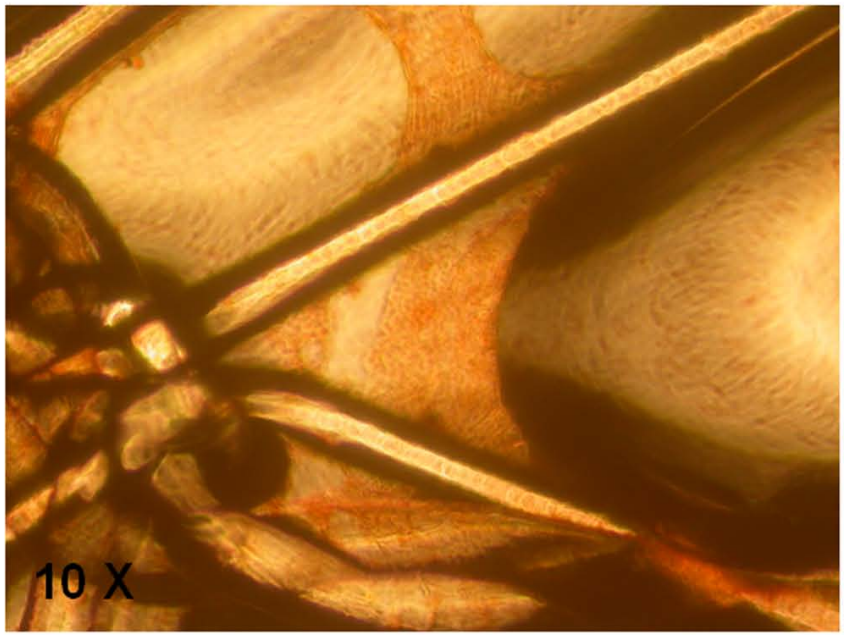


Figure 7

Collagen I

21 days



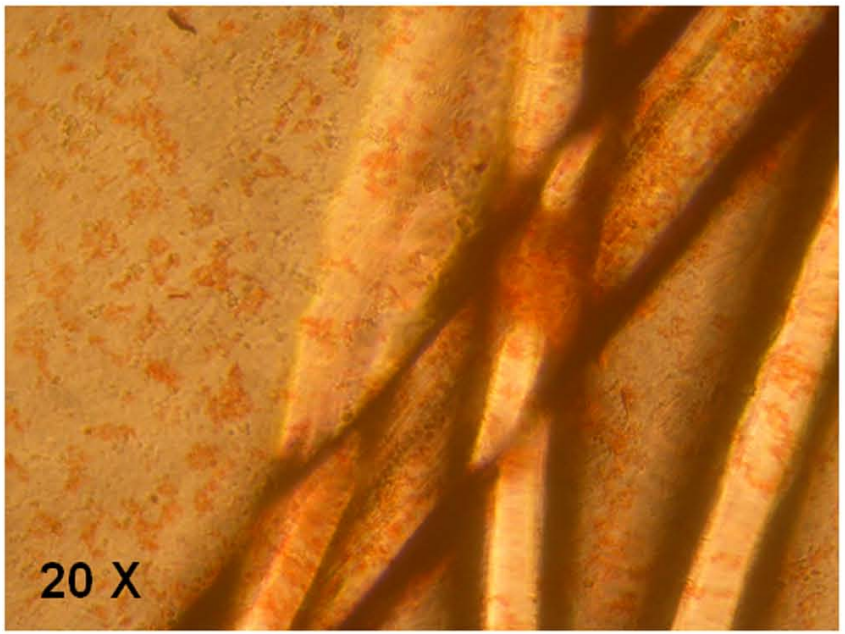


Figure 8

Cytokines